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#9

Advances in Brief

α-Fetoprotein-specific Genetic Immunotherapy for Hepatocellular Carcinoma [1]

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Abstract

The majority of human hepatocellular carcinomas overexpress α-fetoprotein (AFP). Two genetic immunization strategies were used to determine whether AFP could serve as a target for T-cell immune responses. Dendritic cells engineered to express AFP produced potent T-cell responses in mice, as evidenced by the generation of AFP-specific CTLs, cytokine-producing T cells, and protective immunity. AFP plasmid-based immunization generated less potent responses. These novel observations demonstrate that this oncofetal antigen can serve as an effective tumor rejection antigen. This provides a rational, gene therapy-based strategy for this disease, which is responsible for the largest number of cancer-related deaths worldwide.

Introduction

Our improved understanding of antigen processing and presentation by professional antigen-presenting cells, as well as the requirements for induction of T-cell immunity, has spawned the discipline of genetic immunotherapy. DNA-based immunization strategies are capable of generating strong cellular immune responses to a variety of antigens, including tumor antigens.

A major obstacle in developing rational strategies in tumor immunotherapy has been the identification of suitable target tumor antigens. The recent identification of several human melanoma rejection antigens using cytolytic T-cell clones has greatly modified our thinking about self/non-self discrimination and what actually constitutes a genuine tumor antigen (1). Many of these melanoma antigens (MART-1, gp100, and tyrosinase), as well as those associated with other malignancies (carcinoembryonic antigen and prostate-specific antigen), represent normal, nonmutated self proteins (2–4). It is now evident that these normal protein determinants have not induced self-tolerance, can be recognized by the mammalian T-cell repertoire, and, when properly and specifically activated, can mount an effective antitumor response that is essentially an autoimmune response (5–7).

The majority of human HCCs[3] overexpress the oncofetal antigen AFP. This M_r 70,000 glycoprotein, produced at high levels by the fetal liver, is transcriptionally repressed at birth and is present thereafter at low serum levels throughout life (8). Serum AFP measurements play an important role in the diagnosis and monitoring of HCC. We wished to determine whether the murine immune system, despite being exposed to AFP (at even higher relative levels than humans) throughout life, could generate T-cell responses to this oncofetal antigen. Two powerful genetic immunization modalities, genetically engineered DCs and plasmid DNA, were used to generate AFP-specific T-cell responses in a murine model. Both strategies induced AFP-specific immunity, as evidenced by *in vivo* protection against AFP-producing murine tumors and by the stimulation of AFP-specific CTLs and cytokine-producing T cells.

Materials and Methods

Construction of mAFP Expression Vectors. RT-PCR primers were designed to include the entire mAFP coding region, including the signal sequence. The primers used were 5-GCCATGAAGTTGTGGATCACA-3 and 5′-CTCTCTCTCTCTAGATTAAACGCCCAAAGCATCA-3. RT-PCR template total RNA was isolated from Hepa 1-6 cells by the TRIzol (Life Technologies, Inc., Gaithersburg, MD) method. The 1.8-kb *mAFP* cDNA PCR product was initially cloned into pCR3.1neo (Invitrogen, San Diego, CA) according to manufacturer's instructions for T-A cloning to generate stable transfectants. The 1.8-kb *mAFP* cDNA and the 400-bp *MART-1* cDNA (9) were subcloned into expression vector VR1012 (Vical, San Diego, CA) by blunt-end cloning for i.m. immunization. The *mAFP* cDNA was also subcloned into pAC-CMV-pLpA (AdV shuttle vector), which was used to generate the virus AdVmAFP as described previously (10).

Mice and Cell Lines. Female 5–8-week-old C57BL/6 mice (H-2^b) were purchased from The Jackson Laboratory (Bar Harbor, ME) and handled in accordance with the animal care policy of the University of California, Los Angeles. EL4 is a spontaneous murine lymphoma that arose in C57BL/6 mice, and both Hepa 1-6 and BWIC3 are derivatives of BW7756 (11). EL4 and Hepa 1-6 were obtained from the American Type Culture Collection (Rockville, MD), and BWIC3 was obtained from Dr. John Papaconstantinou (University of Texas, Galveston, TX). Murine cell lines were maintained *in vitro* in DMEM (Life Technologies, Inc.) with 10% FCS (Omega Scientific, Tarzana, CA) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemini Bioproducts, Calabasas, CA). EL4(mAFP) was developed by stable transfection of the EL4 cell line with pCR3.1 mAFPneo by lipofection (DMRIE-C; Life Technologies, Inc.). Transfected cells were maintained under constant G418 selection (0.5 mg/ml; Life Technologies, Inc.) in RPMI 1640 (Life Technologies, Inc.) complete media.

Mice were immunized once with 5×10^5 DCs/mouse administered s.c. in the left flank or 100 μg plasmid/mouse administered i.m. in the left anterior tibialis muscle and challenged 14–21 days later with 7.5×10^4 to 5×10^5 EL4(mAFP) cells/mouse on the right flank and EL4 on the left flank. Cells used for tumor challenge were obtained from single cell suspensions of progressively growing tumors in syngeneic mice to avoid the confounding effects of media and serum-derived products on *in vivo* protection studies (9). Cell suspensions were washed extensively and injected into mice in a final volume of 0.2 ml PBS/animal. Tumor incidence and volume were assessed three times weekly using calipers. Data are presented as mean volume ± SE, as described previously (9).

Preparation of DCs and Adenoviral Transduction. DCs were differentiated from murine bone marrow progenitor cells following the method of Inaba *et al.* (12), with modifications (9). Day +8 nonadherent and loosely adherent cells contained DC aggregates with a high level of major MHC class I and II-, B7.1 (CD80)-, B7.2 (CD86)-, CD1d-, CD18-, and CD44-positive cells that were superior stimulators of a mixed lymphocyte reaction (data not shown). *In vitro* cultured DCs were transduced in 15-ml conical tubes (Costar, Acton, MA) in a final volume of 1 ml of RPMI with 2% FCS to which the virus stock was added at a MOI of 100 viral plaque-forming units/DC. Transduction

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; DC, dendritic cell; mAFP, murine AFP; RT-PCR, reverse transcription-PCR; AdV, adenovirus; MOI, multiplicity of infection; IL, interleukin; ELISPOT, enzyme-linked immuno SPOT.

was carried out for 2 h at 37°C, and then the DCs were washed extensively and resuspended in 0.2 ml PBS/animal for injection into mice. Cell counts were determined using a hemocytometer, with viability assessed by trypan blue exclusion. Viability exceeded 95% in all cases.

In Vivo **Monoclonal Antibody Ablation.** CD4 and CD8 T-cell subpopulations were depleted by i.p. injection of protein G-purified hybridoma supernatants. A total of 0.25 mg/mouse/injection of anti-CD8 (clone 2.43; ATCC TIB 210) or anti-CD4 (clone GK1.5; ATCC TIB 207) purified antibody was injected on days 5, 3, and 1 before tumor inoculation and every 6 days thereafter (13). Adequacy of CD4+and CD8+ T-cell depletion was monitored by flow cytometric analysis of splenocytes at the day of tumor challenge using FITC-labeled anti-CD4 antibody (clone CT-CD4; Caltag, Burlingame, CA) and Phycoerythrin-labeled anti-CD8a antibody (Ly-2; clone 53–6.7; PharMingen, San Diego, CA). Ablated mice had a >90% decrease in CD4 and CD8 cells in the spleen after *in vivo* depletion (data not shown).

Cytotoxicity Assays. For *in vitro* microcytotoxicity assays, splenocytes were harvested 10–14 days after the last immunization, depleted of RBCs by hypotonic lysis, restimulated *in vitro* with irradiated EL4(mAFP) cells (25:1 ratio) + 10 units/ml IL-2, and assayed in a standard 4-h chromium release test (9). Samples were tested against their own maximum and spontaneous release for each different condition.

Cytokine Profile by ELISPOT. ELISPOT assays were performed as follows. Briefly, RBC-depleted splenocytes were restimulated *in vitro* by culture with irradiated EL4(mAFP) or EL4 (25:1 responder:stimulator ratios) cells with 10 units/ml IL-2 for 48 h. Restimulated splenocytes were added in duplicate in 3-fold dilutions to 96-well mixed cellulose plates (multiscreen filtration system; Millipore, Bedford, MA) precoated with anti-IFNγ or anti-IL-4 antibody (PharMingen). After a 24-h incubation at 37°C, plates were washed and incubated at 4°C with a secondary biotinylated antibody and then developed the next day by adding freshly prepared substrate buffer. Spots were counted under a dissecting microscope.

RNA Analysis. Total cellular RNA was isolated using TRIzol (Life Technologies, Inc.). Each RNA sample was treated with DNase (Stratagene, La Jolla, CA), reverse-transcribed with random hexamer primers, and then subjected to PCR with either *mAFP*, neomycin resistance (*Neo*), or murine adenine phosphoribosyltransferase (*APRT*)specific PCR primers to confirm the expression of the mAFP and neomycin transgenes and well as semiquantify the cDNA yield in each reverse-transcribed sample. The primers used for *mAFP* were identical to the cloning primers above, and the others were as follows: *APRT*, 5′-ACTCCAGGGGCTTCCTGTTTG-3 and 5′-ATCCACATTGACCACTCTCTG-3′; and *Neo*, 5′-GGTGGAGAGGCTATTCGGCT-3′ and 5′-GATAGAAGGCGATGCGCTGC-3′.

Statistical Analysis. Results of *in vivo* studies are presented as the mean and SE of tumor volumes in each treatment group (9). Mice completely protected from a tumor challenge are presented separately from mice that did develop tumors. Significance is calculated using the *t* test (or the rank sum test in case of failing the Kolmogorov-Smirnov test for normality) also excluding mice that did not develop tumors. Each study included five mice/group and has been repeated at least twice. Tumor appearance and growth to 10 mm in diameter was calculated by the Kaplan-Meier method, and differences between immunized and control mice were calculated by the Mantel-Haenszel test.

Results and Discussion

Generation of mAFP-expressing Plasmid, AdV, and Cell Lines. The 1.8-kb *mAFP* cDNA was isolated from the murine HCC cell line Hepa 1-6 by RT-PCR and subcloned into both a eukaryotic plasmid expression vector, VR1012 (pmAFP), and an E1-deleted replication-incompetent AdV vector (AdVmAFP). In both vectors, the *mAFP* gene is driven by the cytomegalovirus enhancer-promoter (9). A surrogate murine HCC cell line was constructed by stably transfecting the EL4 lymphoma cell line with *mAFP*. This AFP-producing cell line [EL4(mAFP)] thus served as a T-cell target for both *in vivo* and *in vitro* investigations and could be directly compared with the parental, untransfected control, EL4 (Fig. 1*a*). Similar results were observed when another syngeneic murine tumor, B16 melanoma, was stably transfected with *mAFP* in a similar fashion (data not shown).

Genetically Engineered DC-based Immunization. The DC-based AFP immunization strategy proved superior in generating strong immune responses. We recently reported that human DCs transduced with an AdV expressing the human MART-1 melanoma antigen properly process and present the HLA-A2.1-restricted immunodominant peptide and can stimulate MART-1 peptide-specific human T-cell responses *in vitro* (14). We also reported that murine DCs transduced with the MART-1 gene can generate potent MART-1-specific T-cell responses in mice, as evidenced by *in vivo* tumor protection and the generation of MART-1-specific CTLs (9). Therefore, we applied this proven methodology to the putative mAFP tumor antigen. C57BL/6J murine DCs were generated from bone marrow precursors by cell culture in granulocyte macrophage colony-stimulating factor and IL-4 over the course of a week. These DC-enriched cultures had characteristic DC morphology, phenotype, and biology (12, 15). After transduction with AdVmAFP, these DCs expressed mAFP mRNA in a viral dose-dependent manner, as assessed by RT-PCR (Fig. 1*a*). DCs transduced with AdVmAFP were used to immunize naive mice to a subsequent challenge of EL4(mAFP). Mice receiving one or several weekly s.c. immunizations of AdVmAFP/DC were partially or completely protected from a challenge of EL4(mAFP) (Fig. 1*b*) but not EL4 (Fig. 1*c*). Untransduced DCs and DCs transduced with an empty RR5 AdV vector (Fig. *b*) or with the *Escherichia coli* β-*galactosidase* gene (data not shown) were all ineffective in generating protective immunity. We noted in our MART-1/DC model that protection required the participation of both CD8 and CD4 T cells. Likewise, *in vivo* ablation of either T-cell subset completely abrogated AdVmAFP/DC-induced immunity (Fig. 1*d*), effectively ruling out non-T-cell mechanisms and implying that both MHC class I- and II-restricted responses are being generated. The requirement for MHC restriction is further supported by the observation that only AdVmAFP-transduced syngeneic (C57BL/6; H-2^b) DCs but not allogeneic (C3H/Sem/Kam; H-2^k) DCs induced protective immunity (data not shown). Splenocytes from immunized mice contained mAFP-specific (MHC class I-restricted) CTLs, as measured by short-term chromium release assays (Fig. 1*f*) and specifically released the Th1-type cytokine IFN-γ in ELISPOT assays (Fig. 1*e*). Our cumulative experience with this model includes 12 separate experiments using a total of 140 mice, in which 12 of 89 (13.5%) immunized mice were completely protected from tumor challenge with EL4(mAFP) cells during the 40-day observation period ($P = 0.01$, χ^2). Significantly delayed tumor growth was noted in the remainder of immunized mice (time to reach 10 mm in diameter was 14 days compared to 18.5 days in controls; $P < 0.001$). None of the AdVmAFP/DC-immunized mice (even multiply immunized animals) challenged with EL4 cells ($n = 25$) had altered tumor growth compared with controls. We have observed similar but less impressive protection using murine HCC tumors, probably because these cell lines, all of which have been derived from the spontaneous murine HCC line BW7756 and propagated since 1957, do not express detectable levels of MHC class I or II. The only available AFP-producing murine HCCs are BWIC3 and its derivative cell line, Hepa 1-6.

DNA-based Immunization. In a parallel effort, we examined the efficacy of plasmid DNA-based genetic immunotherapy using i.m. injections of AFP plasmid mAFP (pmAFP). Protection with plasmid immunization was less reproducible than with transduced DCs. After receiving as little as one weekly i.m. injection or up to four weekly i.m. injections of pmAFP, naive C57BL/6J mice generally showed some degree of protection from EL4(mAFP), but not after identical immunization with a plasmid encoding the human MART-1 melanoma antigen (pMART; Fig. 2*a*). Mice were not protected from the parental EL4 tumor (Fig. 2*b*). In 10 separate experiments using a total of 104 mice, 4 of 54 (7.4%) immunized mice were completely protected from EL4(mAFP) (P = not significant). In the remainder of these mice, tumor growth delay was somewhat prolonged [time to reach 10 mm in diameter, 17 days (immunized mice) and 19.5 days (control); $P < 0.001$]. Efforts to improve the DNA-based immuniza-

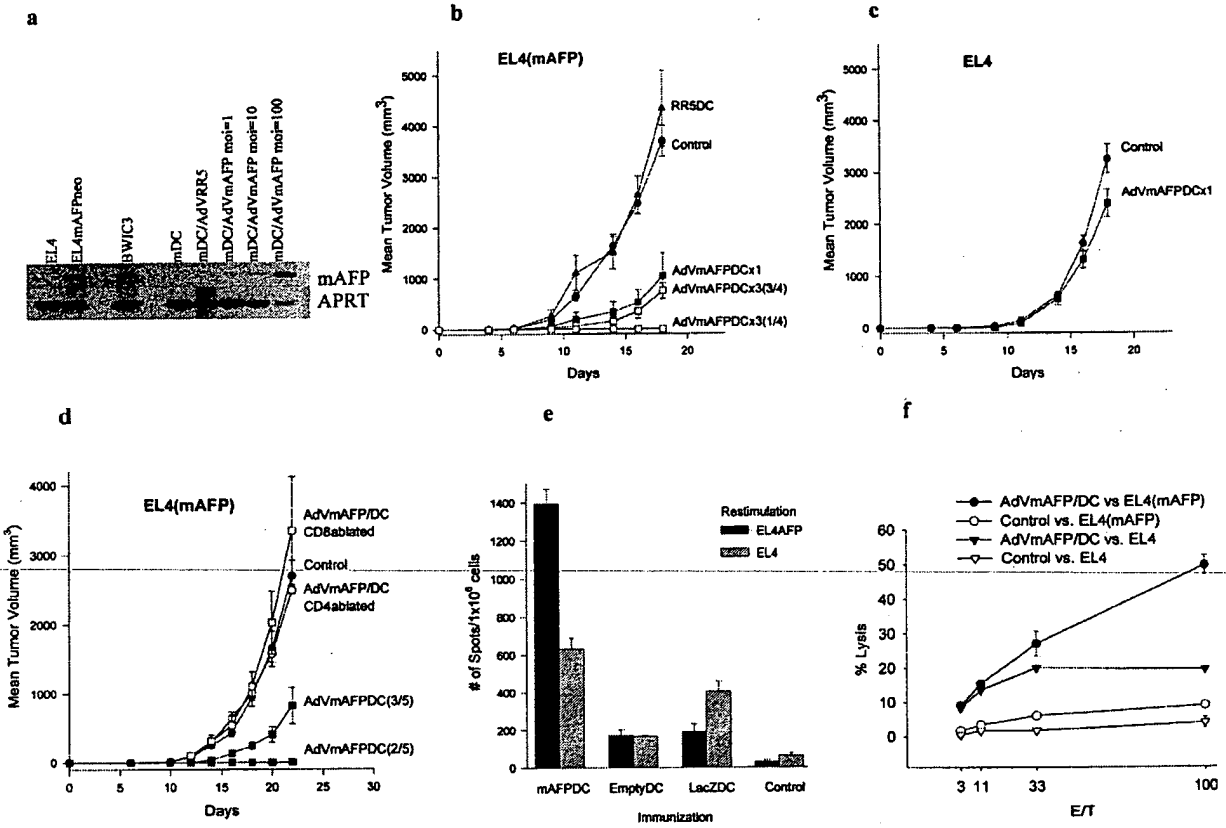

Fig. 1. AdVmAFP/DC generation of AFP-specific immunity. *a*, AFP mRNA expression by DCs transduced with AdVmAFP. DCs were transduced with AdVmAFP at a MOI of 1, 10, and 100, and the relative levels of message expression after 48 h in culture were measured by quantitative RT-PCR using mAFP primers. mAFP is also expressed by the HCC cell line BWIC3 and EL4(mAFP), but not by the EL4 parental line. *b*, AdVmAFP/DC-immunized mice are protected from a challenge with EL4(mAFP) cells. Mice received one (■) or three (□) weekly s.c. injections of 5×10^5 AdVmAFP/DC (MOI = 100) and were challenged 1 week later with 5×10^5 EL4(mAFP) cells. Partial or complete protection was observed compared with untreated controls (●; $P = 0.001$) or mice given DCs transduced with an empty RR5 vector (▲; $P = 0.009$). *c*, AdVmAFP/DC-immunized mice are not protected from the parental EL4 tumor. A parental EL4 tumor (5×10^5 inoculum) challenge grew progressively in control mice (●) and in those mice immunized with AdVmAFP/DC × 1 (■). *d*, AdVmAFP/DC-generated immunity was completely abrogated in mice depleted of CD8 or CD4 T-cell subsets. Control, ●; AdVmAFP/DC, ■; AdVmAFP/DC/CD8 ablated, □; AdVmAFP/DC/CD4 ablated, O. *e*, frequency of IFN-γ-producing immune splenocytes. Splenocytes were retrieved 2 weeks after DC immunization; splenocytes restimulated for 48 h *in vitro* with irradiated EL4(mAFP) or EL4 cells had a higher EL4(mAFP)-stimulated IFN-γ release as measured by ELISPOT assay than did splenocytes from control or empty DC-treated mice. Data are expressed as the number of spots/one million cells. *f*, induction of AFP-specific CTLs after AdVmAFP/DC immunization. Splenocytes were retrieved from mice immunized with AdVmAFP/DC (same conditions as described in *e*) and assayed for lytic activity in a standard 4-h chromium release assay. Splenocytes from AdVmAFP/DC-immunized mice were cytotoxic for EL4(mAFP) cells (●), but not for the parental EL4 cells (▼; $P = 0.003$). Splenocytes from unimmunized mice lysed neither EL4(mAFP) cells (□) nor EL4 cells (itrio).

tion by coinjection of pmAFP with expression plasmids encoding murine IL-12, murine B7.1, murine B7.2, or murine granulocyte macrophage colony-stimulating factor were not successful (data not shown). Draining lymph node lymphocytes (and, less commonly, splenocytes) were cytotoxic for (Fig. 2*c*) and released IFN-γ when restimulated by (Fig. 2*d*) *mAFP*-transfected targets. As with the DC immunizations, only sporadic protection was observed using BWIC3. Because the final immunological pathway for DNA-based immunization is antigen uptake and processing by host antigen-presenting cells, it is not surprising and is consistent with observations by us and others (16–22) that tumor antigen gene-engineered DCs induce stronger immunity.

Treatment Toxicity. Because these efforts represent the generation of an autoimmune response to a self oncofetal antigen, we wished to determine whether the induction of AFP-specific T-cell responses was accompanied by toxicity in these mice. Subjectively, AFP-immunized mice appeared no different from control animals. Standard H&E histological examinations of liver, lung, kidney, and brain from AdVmAFP/DC-immunized mice were interpreted as normal by an experienced surgical pathologist, were indistinguishable from controls, and specifically did not show any features suggestive of an inflammatory or autoimmune response.

These experiments clearly demonstrate that both AFP-transduced DCs and AFP plasmid immunization can generate effective T-cell immune responses *in vivo*. These findings are consistent with the observations made with many other self antigens that have been shown to serve as effective tumor rejection antigens (23, 24). The fact that the T-cell repertoire has not been deleted of these self-reactive clones and can be activated by antigen-engineered DCs is remarkable. Presumably, the overexpression of these self antigens by tumors (MART-1, gp100, MAGE, carcinoembryonic antigen, and now AFP) renders them susceptible to T-cell-based immune responses (25, 26).

This represents the first report that this oncofetal antigen can serve as a target for cell-mediated immune responses. This novel observation provides a solid rationale for developing AFP-directed genetic immunotherapy for the treatment and possible prevention of HCC, a disease responsible for the largest number of cancer deaths with an annual global incidence of 1.2 million (27).

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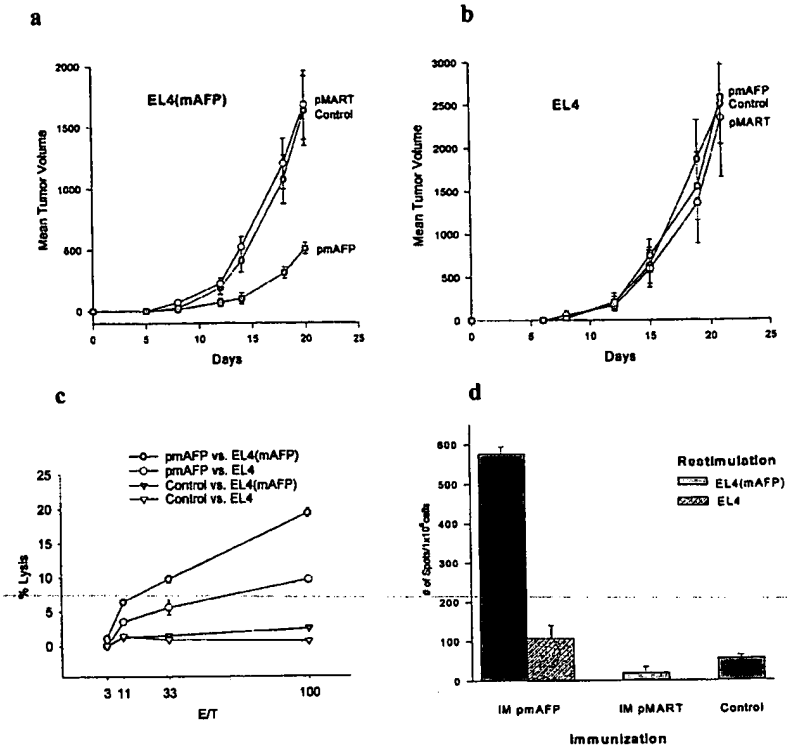

Fig. 2. AFP plasmid-based generation of AFP immunity. *a*, AFP plasmid-immunized mice are partially protected from a challenge with EL4(mAFP) cells. Mice received one i.m. injection of 100 μg of either pmAFP plasmid (□) or a plasmid (pMART) expressing the human MART-1 melanoma cDNA (○) or no treatment (control; ○). Two weeks after immunization, mice were challenged with 7.5×10^4 EL4(mAFP) cells. Delayed tumor growth was noted in AFP plasmid-immunized mice compared with both untreated controls ($P = 0.008$) and mice immunized with pMART ($P = 0.003$). *b*, AFP plasmid-immunized mice are not protected from a challenge with parental EL4 tumor. Mice identical to those in *a* were challenged with parental EL4 cells, and no difference in tumor growth was noted. *c*, induction of AFP-specific CTLs in AFP plasmid-immunized mice. Mice received 2 weekly injections of 100 μg of pmAFP or no treatment. Ipsilateral lymph node lymphocytes were harvested 2 weeks after the final immunization, restimulated for 72 h with irradiated EL4(mAFP) cells, and assayed for lytic activity in a standard 4-h chromium release assay. Lymphocytes from pmAFP-immunized mice lysed EL4(mAFP) (◎) but not EL4 (▽; $P = 0.0002$), whereas lymphocytes from control mice lysed neither target (□ and ▽). *d*, frequency of IFN-γ-producing draining lymph node lymphocytes. Ipsilateral lymph node lymphocytes retrieved from mice immunized with pmAFP (restimulated *in vitro* with EL4(mAFP) or EL4 cells for 48 h) had a higher EL4(mAFP)-stimulated IFN-γ release as measured by ELISPOT assay than control or pMART-treated mice lymphocytes.

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